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BRIEF REVIEW

The SPO1-related bacteriophages

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Abstract A large and diverse group of bacteriophages has been termed 'SPO1-like viruses'. To date, molecular data and genome sequences are available for *Bacillus* phage SPO1 and eight related phages infecting members of other bacterial genera. Many additional bacteriophages have been described as SPO1-related, but very few data are available for most of them. We present an overview of putative 'SPO1-like viruses' and shall discuss the available data in view of the recently proposed expansion of this group of bacteriophages to the tentative subfamily *Spounavirinae*. Characteristics of SPO1-related phages include (a) the host organisms are *Firmicutes*; (b) members are strictly virulent myoviruses; (c) all phages feature common morphological properties; (d) the phage genome consists of a terminally redundant, non-permuted dsDNA molecule of 127–157 kb in size; and (e) phages share considerable amino acid homology. The number of phages isolated consistent with these parameters is large, suggesting a ubiquitous nature of this group of viruses.

The 'O' in the name SPO1 probably represents the letter 'O' from the city of Osaka, where SPO1 was isolated. In the published literature, the number zero '0' has occasionally been used instead, making it necessary to use both forms of the name for literature searches and database mining.

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Introduction

Bacteriophages are classified into families by gross morphology and the nature of their nucleic acid. More than 5,500 bacteriophages have been examined by electron microscopy [9, 11, 12] and the number of morphologically characterized bacteriophages is steadily increasing. Phage morphology is characterized by extreme diversity in a small subset of isometric, filamentous or pleomorphic phages (4%), whereas approximately 96% of all bacteriophages are tailed and belong to the order *Caudovirales*. Bacteriophages of this order are subdivided into three families, the *Myoviridae* (25%), phages with a contractile tail, the *Siphoviridae* (61%) with a non-contractile, flexible tail, and the *Podoviridae* (14%) with a short, non-contractile tail [9, 11]. One especially interesting class of bacteriophages infecting Gram-positive, low G + C-content host bacteria such as *Bacillus*, *Staphylococcus*, *Listeria* and *Enterococcus* comprises the so-called 'SPO1-like' phages. The members of this group of myoviruses are strictly lytic, i.e. virulent. Because of their generally broad host range, they are ideally suited as agents for the biocontrol of pathogenic bacteria and, in recent years, have thus been objects of considerable attention.

The current ICTV genus 'SPO1-like viruses' contains ten *Bacillus* phages and one *Lactobacillus* phage [46]. Until recently, the only genome sequence available was that of phage SPO1 itself [97]. The term 'SPO1-like' has in the past been assigned to a highly diverse group of bacteriophages infecting hosts from different genera. In most cases, this classification is based on morphological criteria

and little or not on molecular data such as nucleotide sequence homologies or the presence of hydroxymethyl uracil in phage DNA. In 2009, a taxonomic proposal was submitted to the ICTV requesting the creation of the subfamily *Spounavirinae* with the genera 'SPO1-like viruses' and 'Twort-like viruses' and eight phage species, to integrate and accommodate the wealth of new molecular information available for these phages. In this proposal, the genus 'SPO1-like viruses' contains phage SPO1, whereas the group 'Twort-like viruses' contains phages Twort, K, G1, P100 and A511. Phages LP65 and φEF24C were classified as orphans within the subfamily [58] (Fig. 1). This proposal is under examination by a special study group chaired by JK. For the purpose of this review and to avoid confusion, we shall use here the designation 'SPO1-related' for all phages previously named 'SPO1-like viruses', including the 'Twort-like viruses' and for all putative and confirmed members of the proposed new subfamily *Spounavirinae*.

In this review, we provide a comprehensive overview of the vast quantity of information available for SPO1 itself and the SPO1-related phages from different genera of Gram-positive bacteria. We present an assessment of the current taxonomic situation and discuss the proposal for resolving the current unsatisfactory taxonomic state of this vast phage group [46]. In addition, we evaluate new members of this group according to the most recent molecular data and genome sequences.

Bacillus subtilis bacteriophage SPO1

Bacteriophage SPO1 was isolated from soil by S. Okubo of the University of Osaka (1964) [75]. SPO1 has been extensively studied for decades (Tables 1, 2). It is a large virulent myovirus of *Bacillus subtilis*, featuring a head with icosahedral geometry (triangulation number $T = 16$) with an average diameter of 84.5 nm (HWA, unpublished results), 87 nm [80] or 108 nm [36], depending on the electron microscopes and measurement methods used (Figs. 2, 3; Table 2), and sharing characteristics with herpes viruses [36]. The long contractile tail consists of a complex baseplate and a 140.3-nm-long and 18.6-nm-wide tail sheath made of stacked disks [79]. The contracted sheath is 63.4 nm long and 26.7 nm wide. The baseplate, which functions as a receptor-recognition device and as a trigger for tail sheath contraction, undergoes structural rearrangement upon contraction [79] (Fig. 2). SPO1 features a double-stranded DNA genome of 145.7 kb in which thymine is fully substituted by 5′-hydroxymethyl uracil (HMU) [74] (Table 1). Its genome sequence has recently been published [97]. The genome features redundant, invariable repeats of 13.185 kb at both ends [96, 97]. The G + C-content of SPO1 is 40 mol% and thus slightly different from the 43.5 mol% G + C of its *Bacillus* host [97]. A total of 204 protein coding sequences and 5 tRNA genes have been annotated. No fewer than 30 genes are present in the terminal redundancy, 24 of them possibly playing a role in host takeover upon infection with SPO1 [96]. The genetic organization of SPO1, transcription and function of the encoded gene products have been analyzed in detail (for a review see ref. [97]). Furthermore, a genetic system defined by conditional lethal mutants exists [76].

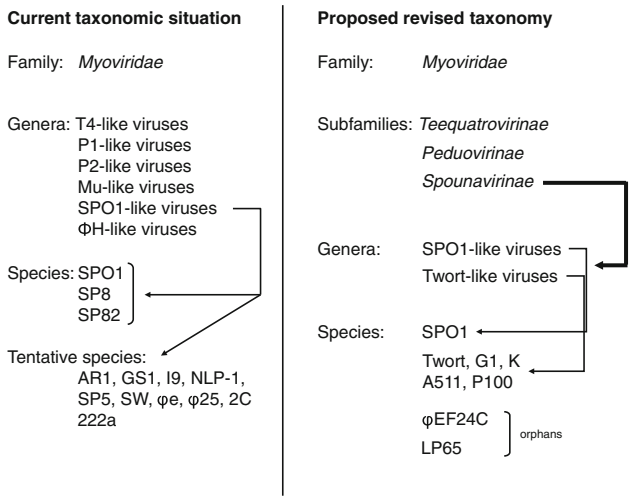

Fig. 1 Comparison of the current taxonomic organization of the family *Myoviridae* and the proposed revised taxonomic scheme [58]

Other *Bacillus* bacteriophages

In the past 50 years, large numbers of *Bacillus*-specific bacteriophages have been isolated and described. One group of closely related, large-tailed *Bacillus subtilis* phages is of special interest because its members share common characteristics with SPO1, are easy to grow and yield high-titer lysates [45]. These include phages SP8, SP82G, φe, 2C and H1 (Table 2; Fig. 2). Additional putative members of the SPO1-related viruses infecting *Bacillus* are φ25, SP50, W.Ph., Bastille and CP-51, and others listed in Tables 2 and 3 (Fig. 2), which will be discussed below. The literature also includes phages SP82 and SP82G, which are claimed to be distinct [45]. Nonetheless, we will only use the designation SP82G in this review.

Bacteriophages SPO1, SP8, SP82G, 2C, H1 and φe have been designated by Hoet et al. [47] as members of the C2 morphotype of *Bacillus* phages. They are described as large myoviruses with a diameter of 85–100 nm, a neck, and a 140–165-nm-long tail sheath. Most phages show conspicuous capsomers on the head, giving the capsid somewhat

Table 1 Characteristics of completely sequenced SPO1-related phages and *Spounavirinae* candidates

Host	Designation	Genome size (kb)	Predicted ORFs	tRNAs	Genome structure	HMU	References
Bacillus subtilis	SPO1	145.747	204	5	Tr, 13.2 kb fixed repeats	Present	[75, 76, 97]
B. cereus	CP-51	~138	~200	2	Fixed ends, ND	Present	This work
Brochothrix thermosphacta	A9	127 ± 1[a]	198	6	Tr, 11 kb fixed repeats	Absent	[52]
Enterococcus faecalis	φEF24C	142.072	221	5	Circularly permuted[b]		[110]
Lactobacillus paracasei	Lb338-1	141.832	199	2	Nonredundant[b]		[15]
L. plantarum	LP65	131.573	165	14	Nonredundant[b]		[29]
Listeria monocytogenes	A511	137.619	199	16	Tr, 3.125 kb repeats	Absent	[53, 61, 111]
L. monocytogenes	P100	131.384[c]	174	18	Tr, ca. 6 kb repeats	Absent	[25, 53]
Staphylococcus aureus	G1	138.715	214	3[d]	ND		[54]
S. aureus	K	127.395[c]	118	3[d]	Tr, ca. 20 kb repeats		[53, 72]
S. hyicus	Twort	130.706	195	1[d]	ND	Absent	[54]

Genome sizes include size of the terminal redundancy (if known)

HMU, 5-hydroxymethyl uracil; kb, kilobase; ND, not determined; Tr, terminally redundant; non-permuted

[a] The A9 genome size has been determined with 1 kb uncertainty due to the presence of a highly repetitive AT-sequence, which could not be fully sequenced. The exact size of the terminal redundancy is unclear, and the genome size is given without redundancy

[b] No data presented by authors

[c] Exact size of terminal redundancy not known- genome length without redundancy is given

[d] tRNA numbers predicted using tRNA-Scan SE [65]

Table 2 Characteristics of SPO1-like *Bacillus* bacteriophages (SPO1-related *sensu stricto*)

Host	Phage	Head diameter (nm)	Tail length (nm)	Capsomers	Double base plate	Genome size (kb)	HMU	References
Bacillus cereus	CP-51	90	160–185	+		~138	+	This work, [100–102, 115, 116]
B. licheniformis	NLP-1	100	130	+			+	[89]
B. subtilis	SPO1	**84.5**–108	140–152	+	+	145.7	+	This work, [75, 97, 107]
	SP8	95–100	157–165	+	+	~150	+	[33, 47, 87, 107]
	SP82G	88–100	162	+	?	~150	+	[45, 47, 77, 107]
	AR1	85	130	+	+		?	[2, 19, 103]
	H1	86	148	+	+	~150	+	This work, [47]
	I9	96	145	+			?	[86]
	SW	101	173				+	[71]
	Vx	100	170				+	[91]
	2C	85–88	125–142	+	?	~150	+	[47, 67, 107]
	φe	90–93	152–180[a]	+		~150	+	[2, 47, 107]
	φ25	75	130	+		~153	+	[2, 45, 59]

Catalase-calibrated measurements are shown in bold

+, present; ?, possibly present; HMU, 5-hydroxymethyl uracil; kb, kilobase; nm, nanometer; ~, approximately

[a] Tail dimensions probably include the base plate and are not comparable to other dimensions in these tables

serrated edges (Fig. 2). SPO1 and SP8 show a tiny collar below the head. In phages SPO1 and SP82G, it was shown that the baseplate rearranges itself into a double-ringed structure upon contraction [45, 79] (Fig. 2). The C2-morphotype phage DNA is approximately 150 kb in size and contains HMU instead of thymine [18, 45, 49]. All phages feature non-permuted genomes with approximately 10% terminal redundancy, shown to be 12 kb in phage C2 [30, 40, 45, 47], and 13 kb in SPO1 [96, 97]. DNA–DNA hybridization experiments indicate considerable sequence homology between all group members, except H1, which was not analyzed [47, 107], while phage φ25 seems to be a more peripheral member of the group [47]. Phage φ25 is described as having a head diameter of 75 nm, a

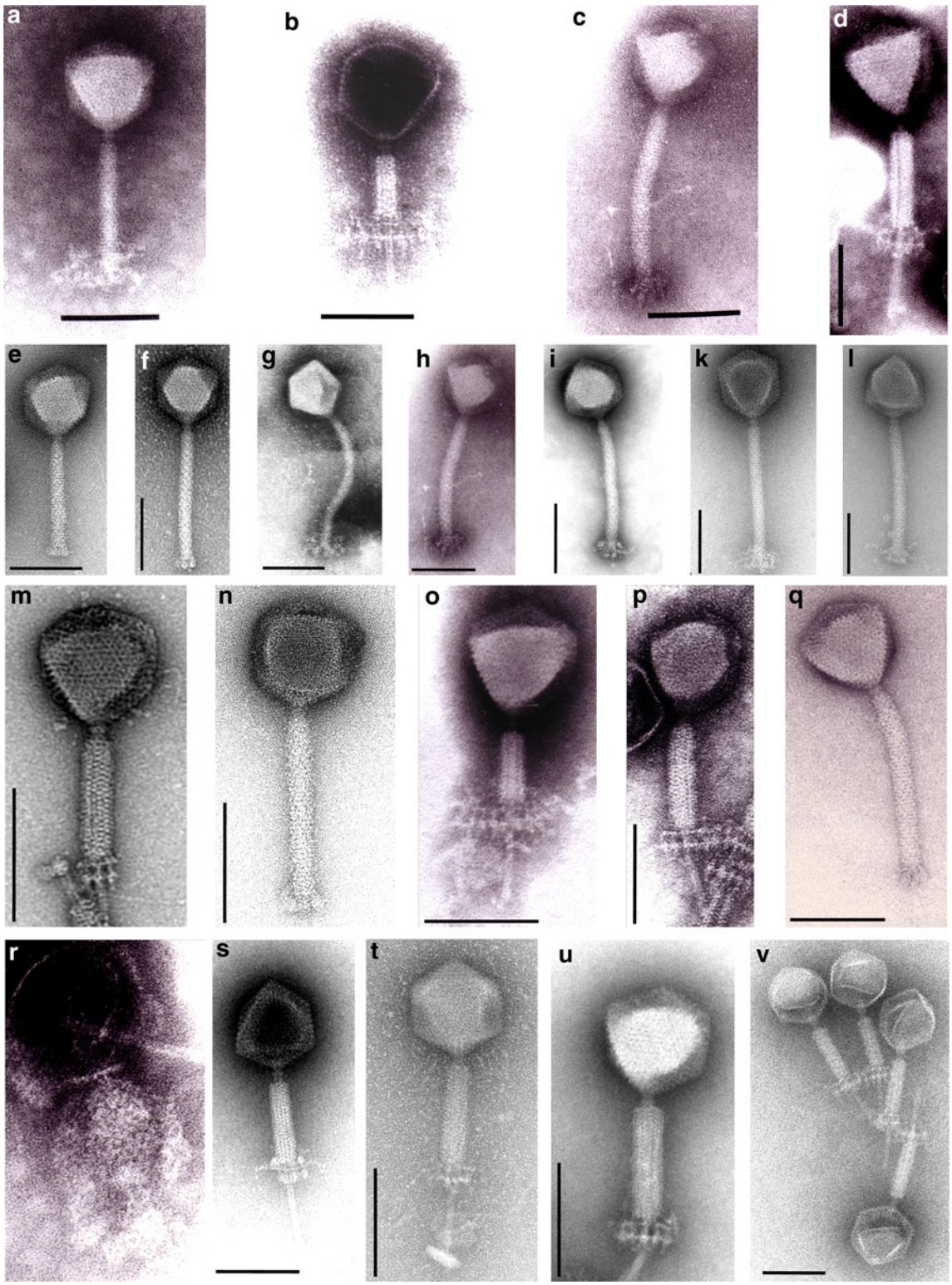

Fig. 2 Transmission electron microscopic images of SPO1 and SPO1-related bacteriophages. **a**, **b** *B. subtilis* phage SPO1 (contracted tail **b**); **c**, **d** *S. hyicus* phage Twort (contracted tail **d**); **e** *B. cereus* phage CP-51; **f** *B. thuringiensis* phage Bastille; **g**, **h**, **i** *S. aureus* phages G1, G4 and K; **k**, **l** *L. monocytogenes* phages P100 and A511; **m** *B. cereus* phage W.Ph. contracted tail; **n** *B. cereus* phage CP-51; **o** *B. subtilis* phage SP8, contracted tail; **p** *B. thuringiensis* phage TP50, contracted tail; **q** *B. thuringiensis* phage DP7; **r** *B. thuringiensis* phage TP50, disintegrated head structure with distinct capsomers visible; **s** *L. monocytogenes* phage P100 contracted tail, tail fibers visible; **t** *L. monocytogenes* phage A511, contracted tail; **u** *S. aureus* phage K, contracted tail; **v** *B. thermosphacta* phage A9, contracted tails. *Bars* represent 100 nm

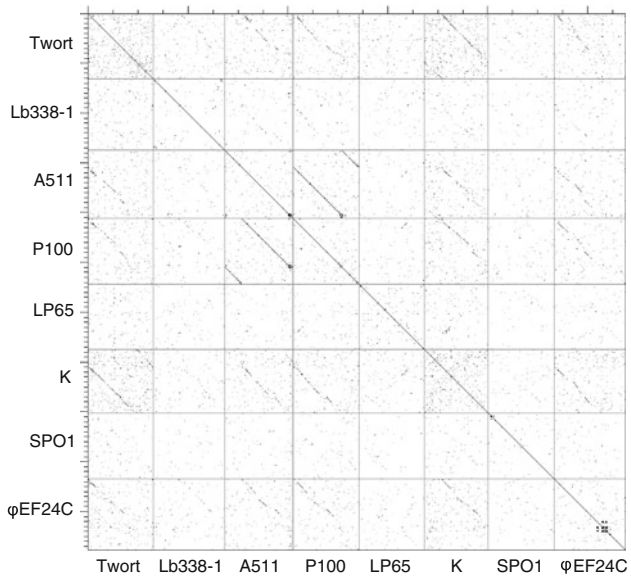

Fig. 3 Dot plot of nucleotide sequences from published SPO1-like phages. The plot was generated using Dotter [94] Linux version 2002 with a sliding window size of 25. The position of phage genomes is indicated on the *X*- and *Y*-axes. Scale of *Y*-axis is 1,000 bp; scale of *Y*-axis is 50 kb. GenBank accession numbers of used the sequences used are: Twort (AY954970), Lb338-1 (FJ822135), A511 (DQ00 3638), P100 (DQ004855), LP65 (AY682195), K (NC_005880), SPO1 (FJ230960), φEF24C (NC_009904)

surprisingly short tail of 130 nm and a HMU-containing genome of approximately 153 kb [45, 59]. As in the other phages, the φ25 baseplate undergoes structural rearrangements to appear as a double structure upon tail contraction [45, 59]. As the head diameter seems to be somewhat small for such a large genome, we suppose that this reflects an error of measurement of 10–20%.

Surprisingly, despite the huge number of SPO1-related bacteriophages isolated from *Bacillus* bacteria, SPO1 is the only phage among them whose genome has been sequenced. We recently undertook the sequencing and characterization of *Bacillus* phage CP-51, which is clearly also SPO1-related (JK, unpublished data) and shares 41% homologous genes with SPO1 (hit-length threshold 100 amino acids, 38–100% identity). Only 17% of gene products are homologous (22–70% identity) to phage Twort. CP-51 was isolated from soil by Curtis B. Thorne in 1968 and was shown to be cold-labile [100]. The head diameter was estimated to be 90 nm, and the tail length was reported as 160–185 nm [7], similar to our own observation (Fig. 2e, n; Table 2). The genome of CP-51 is approximately 138 kb in size and features invariable genome ends (JK et al., in preparation). CP-51 DNA was previously found to contain HMU instead of thymine [116]. We observed that *in silico*-predicted restriction sites do not match experimentally obtained data, thus confirming similar observations by Hoet et al. [47]. CP-51 has been found to transduce genetic markers in *B. cereus* and *B. thuringiensis* [100, 115], a feature not in agreement with the proposed presence of invariable terminal repeats in SPO1-related phages [53, 97]. As the transduction frequency is very low (R. Calendar, personal communication), we assume that the observed infrequent transduction is due to occasional packaging errors by the terminase holoenzyme.

As with SPO1, *Bacillus* phage SP50 exhibits a head diameter of 80–100 nm, but has a much longer tail length of 170–203 nm (Table 3). As SPO1, it exhibits capsomers, and the base plate of the contracted tail appears as a double ring [48]. The genome has been estimated at 166 kb [8, 38, 48, 70, 82, 107] (Table 3). SP50 DNA is non-permuted, does not contain HMU instead of thymine, and the G + C content of 41.6 mol% is slightly higher than the 39 mol% estimated for SP8, SP82G, SPO1, 2C and φe by Truffaut et al. [22, 85, 107]. DNA–DNA hybridization indicates that there are few similarities between SP50 and 2C morphotype phages [107].

Bacillus thuringiensis phage Bastille [8, 63], another putative member of the SPO1-related phages, has similar dimensions [48] (Fig. 2f). Its morphology is indistinguishable from phage Twort, and its protein profile is very similar, although no DNA homology could be found in hybridization experiments [48]. We have recently started to characterize Bastille; preliminary data suggest a genome size of approximately 154 kb. Bastille exhibits the characteristic double baseplate in TEM images of contracted tails [48] (J. Klumpp, unpublished data). Out of the 127 gene products annotated in the preliminary genome sequence, 40% exhibit homology to phage Twort (threshold, 100-amino-acid hit length, identity 33–73%) and 28% to SPO1 (31–89% identity).

Another interesting member is *B. cereus* phage W.Ph. (W. Beyer, University of Hohenheim, Germany). This phage shows a broad host range within the *Bacillus anthracis*, *cereus* and *thuringiensis* group and features other characteristics of SPO1-related phages (Fig. 2m, Table 3). Sequencing and characterization of W.Ph. is underway. Among the 258 gene products annotated in the preliminary genome sequence, 22% are homologous to Twort proteins (threshold, 100-amino-acid hit length, identity 35–78%) and 15% to SPO1 (31–89% identity).

Thirty-six additional *Bacillus* phages have been identified as SPO1- or TP50-like (see Tables 2, 3; Fig. 2) almost exclusively on morphological grounds. They include: *B. cereus* phages gamma, V and S [98], NK, DB [113] and 1, 4, 5, 6, 7, 8, 9, 10 and 12 [14]; *B. mycoides* phage K [31]; *B. pumilus* phage PMJ1 [50]; *B. subtilis* phages TSP-1 and β22 [45, 55, 114], AR1, AR2 and AR3 [2, 19, 103] and I9 [86]; *B. thuringiensis* phages DP-7, PK1 [8], gal1, thu4 [2, 34], Tg8, Tg12, Tg13 and Tg14 [20, 93] and TP50 [3]. Unfortunately, very few data on genome sequence, size and

Table 3 Characteristics of Twort-like *Bacillus* bacteriophages

Host	Phage	Head diameter (nm)	Tail length (nm)	Capsomers	Double base plate	Genome size (kb)	References
Bacillus cereus	gamma, V, S	80–90	190–195				[98]
	NK, DB	92–93	180	+	+	92[a]	[113]
	1, 4, 5, 6, 7, 8, 9, 10, 12	**90**	**200**	+	?		[14]
	W.Ph.	90	200	+	+	152.9	W. Beyer, J. Klumpp unpublished results
B. mycoides	No 1	81	200	+	+		[2, 103]
	K	100	200	?	?		[31]
B. pumilus	PMJ1	92[b]	220[b]				[50]
B. subtilis	AR2, AR3	90–95	180	+	+		[2, 19, 103]
	SP50	80–100	170–203	+	+	~166	[38, 82, 107]
	TSP-1	90	200	+	+		[45, 55]
	β22	?	?	+	+		[44, 45, 114]
B. thuringiensis	Bastille	90	203	+		~154	[48]
	DP7, PK1	90	190	+	+		[8]
	gal1, thu4	80–90	190–200	+	+		[2, 34]
	Tg8, Tg12, Tg14	90	200?	?	?		[20]
	Tg13	90	220[b]	?	?		[20, 93]
	TP33	88	217				[43]
	TP50	89–93	195–203	+	?		[3]

Catalase-calibrated measurements are shown in bold

+, present; ?, not reported or not observed with certainty; HMU, 5-hydroxymethyl uracil; kb, kilobase; nm, nanometer; ~, approximately

[a] DNA size estimated from addition of restriction fragment sizes

[b] Particle size estimated from published electron micrographs (this work)

structure, restriction patterns and protein profiles are available. Therefore, the classification of these phages as SPO1-related remains uncertain from a genomic perspective.

Staphylococcus phages

A large number of bacteriophages infecting *Staphylococcus aureus*, *S. epidermidis*, *S. carnosus*, *S. hyicus* and various other coagulase-negative staphylococci have been described (Table 4). Most of these phages were isolated in the 1960s to 1980s, and some have been employed for phage therapy. Many *Staphylococcus* bacteriophages exhibit characteristics related to those of SPO1, although the data basis for most of them is rather weak.

Bacteriophage Twort, a large myovirus of *Staphylococcus hyicus*, is thought to be the original phage isolated by F. Twort in 1915 in London from a contaminated bacteria culture and deposited at the Institute Pasteur of Paris and later at the Félix d'Hérelle Reference Center for Bacterial Viruses in Quebec, Canada. It is morphologically similar to SPO1 but is described as having a longer tail of 203 nm × 17 nm [48, 112] (Fig. 2c, d; Table 4). Vieu et al. [112] describe a restructuring of the base plate to a double disk after tail contraction and the presence of 5–7 fibrils. In their effort to sequence 27 *Staphylococcus* phages, Kwan et al. [54] classified Twort, together with the closely related phages G1 and K, into genomic group III of *Staphylococcus* bacteriophages. The genome sequences of K and G1 are nearly identical (90% nucleotide identity), whereas phages Twort and K and Twort and G1, respectively, share approximately 50% nucleotide identity [54]. The Twort genome was determined to be 130.7 kb in size and features a G + C content of 30.6 mol% [54], with no modified bases [2] (Table 1). Phage K, the endolysin of which is successfully employed in control of *S. aureus* and *S. epidermidis* isolates [27, 73], was sequenced and characterized by O'Flaherty et al. [72] in 2004 and exhibits slightly larger dimensions than phage Twort (Fig. 2i; Table 4). The baseplate undergoes a structural rearrangement during contraction, exhibiting the typical double disk morphology. Phage K possesses six tail fibers and six tail spikes attached to the baseplate [53]. The 127.4-kb unit genome [72, 83, 84] probably features an additional terminal redundancy as large as 20 kb [53] (Table 1). No indication of HMU substitution could be found in phage K by restriction

Table 4 Characteristics of Twort-like *Staphylococcus* bacteriophages

Host	Phage	Head diameter (nm)	Tail length (nm)	Capsomers	Double base plate	Genome size (kb)	References
Staphylococcus aureus	G1, G4	90	200	+	+	138.7	This work, [54]
	ISP	90	175	+	?	140	[69]
	K	66–94	191–219	+	+	127.4	[53, 54, 72, 83]
	P1	80	215	+	+		[23, 88]
	P14, Muscae	Similar to Twort	Similar to Twort	?	?		[88]
	P2, P3, P4, P8, P9, P10	86–91[a]	181–240[b]	+	+		[2, 5, 103]
	1623	80–90	225–235[b]	+	+		[2, 103]
	06, 40, 58	75	170	+	+		[4, 5, 68]
	S_b-1	70–80	180	+	+		[5, 17, 28]
	S3K, 119, 130, 200	75	190–200	+	+		[5, 56]
	φ812	92	209		+	~146.5[c]	[78]
	U16, 131	Similar to φ812	Similar to φ812	?	?		[24, 78, 90, 105]
S. carnosus	φSK311	70	200			~141	[39, 78]
S. coagulase	Ph5, Ph9, Ph10, Ph13	107–127 (PT)	203–245[b] (PT)	+	+		[5, 104]
		80–113 (UF)	179–265[b] (UF)				
S. epidermidis	RG	95	115	?	+		[92]
S. hyicus	Twort	**90**	**203**	+	+	130.7	[2, 4, 48, 56, 103, 112]
		75–105	190–200				

Catalase-calibrated measurements are shown in bold

+, present; ?, not observed with certainty; HMU, 5-hydroxymethyl uracil; kb, kilobase; nm, nanometer; ~, approximately

[a] Particle size estimated from published electron micrographs (this work)

[b] Tail dimensions probably include the base plate and are not comparable to other dimensions in these tables

[c] DNA size estimated from addition of restriction fragment sizes

profiling [47, 53]. A *S. aureus* phage, isolated in the beginning of the last century in Georgia, named ISP, was recently characterized in an attempt to develop a phage cocktail for treatment of humans [69]. It is a broad-host-range virulent phage with a head diameter of 90 nm, a tail length of 175 nm and a genome of 140 kb in size. The nucleic acid sequence of ISP is almost identical to that of phage G1, and capsomer-like structures are visible in its electron microscopic images [69].

Phage φ812, closely related to K and G1, probably also falls into this group of phages, according to its dimensions and genome size [37, 78]. It was demonstrated that the φ812 genome is devoid of cohesive ends and features approximately 20 kb of sequence information in addition to the sequenced genome size. Thus, it is likely that φ812 possesses invariable terminal repeats of 20 kb, as proposed for phage K [53, 78]. The same holds true for phage G1, although no experimental proof is available. Twort, K, G1 and φ812 are closely related to another cluster of phages, namely U16, 131 and φSK311 [39, 78, 105]. The restriction map of φ812 was shown to be almost identical to *Staphylococcus carnosus* phage φSK311 [78], thus confirming the relationship on the molecular (species) level. Interestingly, the restriction profiles differ only in the terminal genome fragments. The φSK311 genome and the genomes of the polyvalent phages U16 and 131 were also shown to be devoid of cohesive ends. U16 and 131 were shown to exhibit a high level of homology to φ812 by restriction analysis [53, 78]. φSK311 is a large, broad-host-range, polyvalent phage with tail fibers and spikes [39] (Table 4). The few data available for phages 131 and U16 confirm essentially their relationship with φ812, φSK311 and other related staphylococcal phages. U16 features a broad host range, adsorbing to 132 tested *Staphylococcus* strains [24, 90, 105].

Another large group of 25 phages, said to be similar to phages φ812 and Twort and probably SPO1-related [2, 78], comprises the following (Table 4): P1 [23], P14, S3K and Muscae [56, 88], all four being original members of the group D bacteriophages described by Rosenblum and Tyrone [88]; G4 (this work, Fig. 2h) [78]; S_b-1 [17, 28]; 06, 40 and 58 [68]; RG, Ph5, Ph9, Ph10, Ph13, 06, 40, 58, 200, 119, 130, P2, P3, P4, P8, P9, P10 and 1623 [2, 56, 92, 103, 105]. The last 23 phages are described as having clearly visible capsomers and a double base plate. In a

Table 5 Characteristics of Twort-like bacteriophages of other bacteria

Host	Phage	Head diameter (nm)	Tail length (nm)	Capsomers	Double base plate	Genome size (kb)	References
Brochothrix thermosphacta	A6, A8, A9, A19, A20	**89**	160–**171**	+	+	127 (A9 only)	[6, 41, 52]
Enterococcus faecalis	φEF24C	93	204–208	?	+	142.1	[109, 110]
E. sp.	1	90	203	+	+	~133	[48]
	2, 4, 41, 867	95	180–200	+	+		[4, 32]
Lactobacillus plantarum	fri	90	203	+	+	~95[a]	[48]
	LP65	85[b]	193	+	+	131.6	[29]
L. paracasei	Lb338-1	85	200	?	?	142	[15]
Listeria monocytogenes	A511	87	199	+	+	137.6[b]	[53, 118]
	P100	89	198	+	+	~137[b]	[25, 53]
Streptococcus lactis	c10III	70	180	+	+		[4, 51]
	RZh	86[c]–90	187[d]–205	+	+		[51, 99, 103]
Tetragenococcus (Pediococcus) halophilus	Φ7116	87–96	200	+	+		[108]

Catalase-calibrated measurements are shown in bold

+, present; ?, not observed with certainty; HMU, 5-hydroxymethyl uracil; kb, kilobase; nm, nanometer; ~, approximately

[a] DNA size estimated from addition of restriction fragment sizes

[b] DNA size including terminal redundancy [53]

[c] Particle size estimated from published electron micrographs (this work)

[d] Tail dimensions probably include the base plate and are not comparable to other dimensions in these tables

general way, phages of the same serological group were found to share a high degree of DNA homology (43% and more), as estimated by DNA–DNA hybridization [24]. Most of the phages presented here fall into the serological group D [24], and little information is available for them, while no data are available for phages A/3, A/5, X, PK [81] A, EW, J10, J11, K1 and K2 [24]. They are proposed to be 'SPO1-like' by Pantucek et al. [78] but are not discussed here.

Listeria and *Brochothrix* phages

The genera *Listeria* and *Brochothrix* both belong to the family *Listeriaceae* and are closely related [66, 95]. Most of the known *Listeria* bacteriophages are siphoviruses [35, 60]. Phages B054, A511 and P100 are the only sequenced exceptions [25, 35, 53, 62]. Both A511 and P100, which are large, virulent, broad-host-range myoviruses of *Listeria monocytogenes*, are morphologically indistinguishable (Fig. 2k, l, s, t) and have been classified as being 'SPO1-like' [25, 53, 61, 111, 118] (Table 5). We were able to visualize the presence of a double baseplate in contracted tails and a sixfold symmetry in tail fibers and tail spikes for both phages [53] (Fig. 2s, t). The genome of A511 is 137.6 kb in size, including a 3.1-kb segment of invariable terminal repeats. The genome of P100 is slightly smaller, exhibiting a size of 131.4 kb [25], and it has additional invariable repeats of approximately 6 kb [53] (Table 1). No evidence for the presence of HMU could be obtained [47, 53].

Until recently, no bacteriophages infecting bacteria of the genus *Brochothrix* have been sequenced. The few known *B. thermosphacta* phages have been grouped into three morphologically distinct groups [6, 41]. Species A19 contains five phages named A6, A8, A9, A19 and A20 (Table 5). Members of A19 have been described as being morphologically similar to phages fri, SP10, SP50, Twort and RZh [1, 2]. Phage A9 features the typical SPO1 morphology of a large myovirus, with a head diameter of 89 nm and a 160–171-nm-long contractile tail [6, 52] (Fig. 2v; Table 5). The distal tail structure of phage A9 has been shown to undergo structural rearrangement upon contraction, exhibiting the typical double baseplate seen in many SPO1-related phages [6] (Fig. 2v). The 127-kb genome features additional invariable terminal repeats of 11 kb [52] and encodes a total of 198 gene products, many of which exhibit high amino acid homology to other SPO1-related phages [52] (Table 1). Among these, 27% show homology to Twort gene products (threshold, 100-amino-acid hit length, identity 28–75%) and 19% to SPO1 (26–89% identity).

Lactobacillus phages

Many bacteriophages infecting *Lactobacillus* have been described, mainly because of their ability to disrupt fermentation processes in the dairy industry. Most of them are siphoviruses, while three bacteriophages have historically been proposed to be 'SPO1-like viruses' (Table 5). Molecular data are available for *L. plantarum* phage LP65 [29], isolated from a salami fermentation culture, and for *L. paracasei* phage Lb338-1 isolated from sewage [15]. Both are similar in dimensions (Table 5). Chibani-Chennoufi et al. [29] published electron images of LP65 that clearly show that the distal end of the tail undergoes a rearrangement upon contraction, resulting in the characteristic double baseplate. Tail spikes and a tail fiber are visible. LP65 contains a 131.6-kb genome with 165 annotated open reading frames [29], whereas the Lb338-1 genome is slightly larger (142 kb in size), and 199 annotated putative open reading frames are discernible [15] (Table 1). LP65 is said to have a 'nonredundant' genome, although no supporting data are published [29]. The genome structure of phage Lb338-1 is currently under investigation.

Another SPO1-related bacteriophage of *Lactobacillus* has been described, but its molecular characteristics are incomplete. Phage fri, isolated from a meat starter culture [48, 106], exhibits a 90-nm head diameter, a 203-nm-long tail and tail fibers. Conspicuous capsomers and a double base plate are present [2] (Table 5). Phage fri DNA features a restriction profile different from the profile of *L. plantarum* phage LP65 [29]. This suggests few nucleic acid homologies between these two bacteriophages, although they can both be propagated on the same host (*L. plantarum* LP65). Also, no similarities between LP65 and fri in SDS-PAGE of virion proteins could be found [29].

SPO1-related phages of other bacteria

Eight additional SPO1-related bacteriophages infecting members of various other bacterial genera have been described. They include *Streptococcus* phage RZh, *Enterococcus* phages φEF24C, 1, 2, 4, 41, 867 and *Tetragenococcus* (*Pediococcus*) *halophilus* phage Φ7116 (Table 5).

Streptococcus phage RZh was reported to be 'SPO1-like', but the phage has probably been lost. RZh is described to feature an apparent morphological relationship to phages Twort and SP50, exhibiting the characteristic head morphology, conspicuous capsomers and a double base plate [1, 2, 48, 99, 103].

Enterococcus faecalis phage φEF24C was recently isolated in Japan [109]. It features a broad host range among the enterococci and exhibits a strictly lytic lifestyle. It seems to be especially useful as therapeutic agent against vancomycin-resistant *Enterococcus*. Electron microscopic images show φEF24C to possess the typical morphology of SPO1-related phages [109] (Table 5). Virion proteins exhibit similarity to other SPO1-related phages. The φEF24C genome was sequenced, is 142.1 bp in size and encodes 221 open reading frames featuring homologies to proteins of phages K, G1, Twort, LP65 and P100 [110] (Table 1). Uchiyama et al. describe the φEF24C genome to be 'circularly permuted', but no evidence for this observation is presented [110]. This situation clearly needs further investigation, as it conflicts with previous data on SPO1-related phages.

Another *Enterococcus* phage, unfortunately named 1 ('one'), is morphologically identical to phages Twort, SP50, fri and Bastille [48]. Its genome size is estimated to be 133 kb, and phage 1 DNA does not produce restriction profiles similar to those of phages Twort, SP50, fri or Bastille. Protein profiles showed similarity in structural proteins to phage Twort [48]. No further data are available for this phage, and its classification as SPO1-related remains uncertain.

Conclusions

It is a challenging task to compare the various proposed SPO1-related phages, since no complete set of criteria besides morphological data is available for all candidates. Among the sequenced SPO1-related phages, published genomes show the same overall organization into functional modules. However, nucleotide homology between the members of this group is limited (Fig. 3). Clear sequence homology and synteny are visible between the closely related *Listeria* phages A511 and P100, as well as between *Staphylococcus* phages K and Twort and φEF24C of *Enterococcus*. Thus, these five phages seem to form a more closely related group than phages LP65, Lb338-1 and SPO1. Remarkably, SPO1 is seemingly unrelated at the nucleotide level to the other members of the group.

The predicted proteins of the draft genomes of phages A9 and CP-51 have been compared to those of the nine sequenced phages. Blastp and Blastx analyses [16] demonstrate a relatedness of predicted gene products of all phages, with sequence identities ranging from approximately 20–90%, but mainly restricted to structural proteins of the phage virion and some to DNA-replication-related proteins [15, 29, 53, 58, 110]. *Bacillus* phage CP-51 is the closest SPO1 relative, as indicated by strong amino acid homologies in subsets of both late and early gene products. Figure 4a depicts a phylogenetic tree calculated from ClustalW alignment of protein sequences of phage large terminase subunits. This analysis gives a measure of the

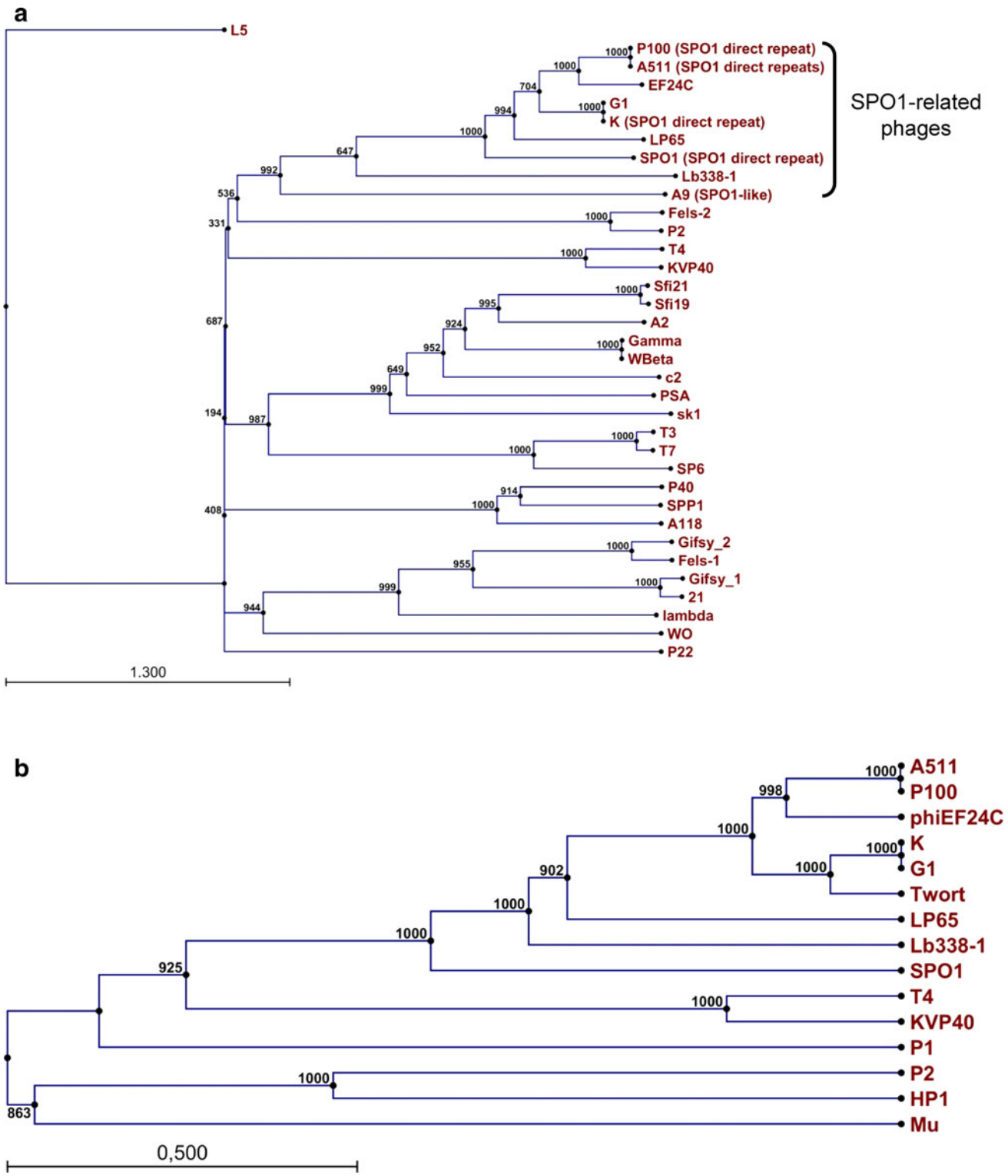

Fig. 4 **a** Phylogenetic tree calculated from ClustalW alignments of large terminase subunit sequences from SPO1-like phages and a selection of phages with known packaging mechanism [26] using the neighbor-joining method and 1,000 bootstrap replicates. Numbers of successful bootstrap replicates are indicated. **b** Phylogenetic tree calculated from ClustalW alignments of the major capsid protein of SPO1-like phages and unrelated phages. The tree was constructed using the UPGMA method and 1,000 bootstrap replicates

similarity between DNA packaging mechanisms by the terminase holoenzyme [26, 48]. It is obvious that even members with a packaging strategy (LP65, Lb338-1, and φEF24C) that is supposedly different from that of phage SPO1 cluster nicely with SPO1 and related phages, thus indicating a conflict between the presented data on the packaging mechanism and the *in silico*-predicted packaging mechanism. This apparent contradiction needs to be resolved. However, the phylogenetic distance of phage A9 from the other compared phages is even greater than in the case of SPO1. This may be attributed to the fact that the A9 large terminase subunit is encoded by three genes, while only the product of the largest orf is compared here. A phylogenetic tree calculated from alignments of the major capsid protein sequences (Fig. 4b) provides a measure of general structural relatedness between the phages compared. All of the phages analyzed cluster in the same tree branch separately from non-SPO1-related phages used for comparison, featuring supportive bootstrap values that confirm the morphological distinctness of SPO1-related phages. Phages SPO1, LP65 and Lb338-1 seem to be less closely related to the rest of the phages, forming a split branch containing A511, P100 and φEF24C on one side and K, G1 and Twort on the other side (Fig. 4b). In conclusion, the SPO1-related phages sequenced to date share undeniable homology in their structural and DNA-packaging protein sequences. This homology is the genetic basis for the observed similarities in morphology and also indicates a shared mode of DNA packaging.

In order to advance the classification of members of the order *Caudovirales*, Lavigne and coworkers [57, 58] used whole-proteome data to group 102 myoviruses for which full genomic data are available. Three subfamilies, including the SPO1-related phages, here named *Spounavirinae*, and eight new independent phage species within the subfamily were proposed (Fig. 1). We used our data on major capsid protein (MCP) [15, 21, 42] and large terminase subunit (TerL) phylogenic clustering [26] to further investigate the validity of giving SPO1-related phages the rank of a *Myoviridae* subfamily [58]. As is obvious from Fig. 4a, b, SPO1-like phages cluster closely together with respect to structural similarities (MCP clustering) and mode of DNA packaging (TerL clustering) and are clearly separated from the other phages. Support for the proposed subdivision of the new subfamily *Spounavirinae* into the 'SPO1-like viruses' and 'Twort-like viruses' [58] stems from whole-genome dot plots (Fig. 3), which indicate that SPO1 is unrelated to the other sequenced group members on the nucleotide level and forms a division of its own. Phage LP65, classified as an orphan by Lavigne et al., seems equally detached from the other phages; however, the other orphan, φEF24C, is somewhat collinear with phages Twort, A511, P100 and K at the nucleotide level (Fig. 3). The same picture is seen in MCP phylogeny, where φEF24C clusters closely with A511 and P100. The *Lactobacillus* phages LP65 and Lb338-1 seem to be more distantly related to these. Lavigne et al. [58] used a threshold of 40 and 20% of overall related proteins for genus and subfamily boundaries, respectively, to classify phages SPO1, Twort, K, G1, A511, P100, LP65 and φEF24C. If we apply these values, then the incompletely characterized SPO1-related phages A9, W.Ph., Bastille and CP-51 can be assigned to the proposed subfamily *Spounavirinae* (W.Ph. and A9), or further differentiated into the genera 'SPO1-like viruses' (CP-51) and 'Twort-like viruses' (Bastille) (Table 2).

When we extrapolate from the presented evidence, essentially in accordance with the (mainly) proteome-based clustering of Lavigne et al. [58], we are able to propose the following common characteristics for all members of the proposed subfamily *Spounavirinae*:

1. Genome size and structure: A ds genome of 127–157 kb in size is indicative of a spounavirus. Furthermore, invariable terminal repeats of 3–20 kb in size have been described for SPO1 [96] and a number of related phages (C2, K, P100 and A511, SP82G) [30, 53, 77] and suspected for others (SP8, H1, SP50 and φe) [22, 30, 40, 45, 47, 85]. It remains to be determined if all phages of this subfamily feature this genome structure. However, the exact resolution of a terminally redundant structure by pulsed-field gel electrophoresis, restriction profiling, Bal31 nuclease digests and/or TEM partial denaturation mapping [53, 64, 117] is both time-consuming and labour-intensive.
2. A generally conserved genome organization with considerable amino acid homologies, but not necessarily high nucleotide sequence homologies. Comparing SPO1-related phages, mainly virion proteins but also a number of DNA replication enzymes share sequence similarity (Figs. 3, 4). This degree of amino acid homology may be used to estimate the relatedness between the compared bacteriophages [58].
3. Hosts belong to the low-G + C-branch of the Gram-positive bacteria (*Firmicutes*). To date, SPO1-like phages have been described for the following bacterial genera: *Bacillus*, *Brochothrix, Enterococcus, Lactobacillus*, *Listeria, Staphylococcus*, *Streptococcus* and *Tetragenococcus*.
4. Strictly virulent lifestyle and a generally broad host range.
5. Morphological observations: All members of the proposed subfamily *Spounavirinae* are myoviruses with isometric heads of approximately 75–100 nm in diameter and a long, more or less rigid contractile tail of approximately 140–220 nm in length (Tables 2, 3,

4, 5). Together with the presence of capsomers, tail fibers and tail spikes indicating sixfold tail symmetry, these are features of a SPO1-related phage. Most SPO1-related phages exhibit a double baseplate upon tail contraction (Fig. 2; Table 2). The bacteriophage head size is directly correlated with the maximum size of the genome it is able to accommodate, as the lateral length of an icosahedral structure determines its volume. In phages SPO1, A511, P100, K, φ812, LP65, Lb338-1, φEF24C and CP-51, a 130–150-kb genome is usually accompanied by a head diameter of approx. 90–100 nm. Thus, the dimensions of the head diameter (and most likely the tail length) of phages φSK311, φ25 and others are probably inaccurate by 10–20%, and the estimate for the genome size of phage fri (95 kb) seems too small for its large head diameter (90 nm). Such ambiguities may be explained by the use of different electron microscopes and stains and the absence of (or faulty) magnification calibration [3, 13]. For example, the tail length of phage SP50 has been reported to be 170 and 203 nm [38, 48]). Differences in genome size may be attributed to different protocols used for DNA extraction and genome size estimation. Some older estimates of bacteriophage genome sizes have been produced by the simple addition of fragment lengths in restriction patterns. An interesting morphological criterion is the presence of visible capsomers in the heads of SPO1 and its relatives. Visible capsomers are extremely rare among tailed phages but have also been observed in *Salmonella* phage P22 [10] and the unrelated, uracil-containing *Bacillus subtilis* myovirus PBS1 [46] (Table 2). Figure 2r shows a disintegrated head of phage TP50 and the individual capsomers.

6. Substitution of thymine in the phage DNA by HMU seems to be widespread among (but restricted to) SPO1-related viruses of *Bacillus* (Table 2) [2]. To date, no indication for the presence of this unusual base has been reported for bacteriophages of other bacterial species. However, it should be mentioned that the presence of HMU is not detectable by sequencing reactions, and most researchers may have overlooked it. One indication for the presence of unusual bases is the fact that restriction profiles do not match *in silico* predictions [47]. The same holds true for identification of gene products, which indicates the presence of DNA modifications. For *Listeria* phages A511 and P100, *Staphylococcus* phage K and *Brochothrix* phage A9, we were able to demonstrate that *in silico* prediction perfectly matched restriction profiles, and no HMU biosynthesis genes are present in the genomes; thus, HMU modification in these phages is unlikely. Other indications are an increase in buoyant density of the phage DNA compared to non-modified DNA in CsCl gradient centrifugation and a modification of the DNA melting point, both of which are rarely determined nowadays. However, positive proof of the presence of HMU requires the use of chromatography techniques [89, 116] or mass spectrometry.

Although many SPO1-related phages have been described, molecular data are scarce for most of them. Consequently, SPO1-related phages have been identified and grouped primarily based on morphological criteria, which alone are no longer sufficient for classification. To revise this group of phages and upgrade it to a subfamily within the ICTV classification of viruses, we propose to list only fully characterized and sequenced phages as full members and to divide them into (a) 'SPO1-like viruses' with relatively short tails, HMU-containing genomes and protein homologies to SPO1; and (b) 'Twort-like viruses' with longer tails, unmodified genomes and homologies to phage Twort. This subfamily includes: SPO1, Twort, K, G1, A511, P100, φEF24C, LP65 and Lb338-1. Phages A9, CP-51, Bastille, W.Ph. and φ812 are likewise valid members of the subfamily but await final confirmation through complete genome sequencing. All other SPO1-related phages should be listed as tentative members of the subfamily or simply as myoviruses related to the proposed subfamily *Spounavirinae* until further experimental data become available. This review shows the extent and prevalence of SPO1-related phages and their ubiquitous nature.

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